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Protein Kinase C Activators Enhance Transmission at the Squid Giant Synapse

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Abstract. We have examined the possible role of protein kinase C in synaptic transmission by asking whether agents that activate protein kinase C affect transmission at the squid giant synapse. Several phorbol esters and a synthetic diacylglycerol that activates the kinase produced a substantial enhancement of transmission at the squid synapse, while structurally related compounds incapable of activating the kinase did not affect transmission. These agents enhanced both postsynaptic potentials and postsynaptic currents, revealing that they were not enhancing transmission exclusively by increasing postsynaptic input resistance. The increase in transmission produced by phorbol esters was either irreversible or reversed over a time course of one hour or longer. Kinase C activators also enhanced transmission at other synapses in the squid stellate ganglion. Our results are consistent with a general role for protein kinase C in synaptic transmission and indicate that the squid giant synapse is a favorable experimental system for further elucidation of the specific function of kinase C at synapses.

Introduction

Protein kinase C (PKC) is a family of calcium-sensitive, phospholipid-dependent protein kinases (Nishizuka, 1984; Coussens *et al.*, 1986; Knopf *et al.*, 1986; Jaken and Kiley, 1987; Ono *et al.*, 1987). Although PKC is found in high concentrations in many nervous systems, including the mammalian brain (Nairn *et al.*, 1985), its physiological functions are largely unknown. The high concentrations of PKC present in presynaptic terminals (Kikkawa *et al.*, 1982; Wu *et al.*, 1982; Girard *et al.*, 1985; Unver *et al.*, 1986) suggests that this enzyme plays a role in synaptic transmission.

Kinase C could play a role in mediating or regulating neurotransmitter release (Augustine *et al.*, 1987). Because activation of PKC enhances secretion from a variety of non-neural cells (Knight and Baker, 1983; Pozzan *et al.*, 1984; Pocotte *et al.*, 1985), PKC may be a necessary component of the molecular apparatus responsible for mediating exocytosis (Baker, 1984). Consistent with such a proposal, transmission at the guinea pig ileum (Tanaka *et al.*, 1984), the frog neuromuscular junction (Publicover, 1985; Eusebi *et al.*, 1986; Haimann *et al.*, 1987; Shapira *et al.*, 1987), and certain synapses in the hippocampus (Malenka *et al.*, 1986, 1987) is potentiated by activators of PKC.

We have attempted to address the role of PKC in synaptic transmission by asking whether agents that activate PKC alter transmission at the squid giant synapse. The large size of the presynaptic terminal of this synapse makes it unusually suitable for detailed analysis of the physiological mechanisms underlying synaptic transmission (Llinas, 1982; Augustine *et al.*, 1988). Protein kinase C is also found in squid nerve terminals (Unver *et al.*, 1986), making a potential involvement of PKC in transmission at the giant synapse more plausible. We have found that agents that activate PKC dramatically enhance transmission at the giant synapse and at other synapses in the squid stellate ganglion. These observations provide further evidence in support of a general role for PKC in synaptic transmission and pave the way for a detailed analysis of the intracellular mechanisms that permit PKC to enhance synaptic transmission. A preliminary report of some of this work has appeared (Osses *et al.*, 1986).

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Materials and Methods

Stellate ganglia of the squids *Loligo pealei* and *L. opalescens* were isolated and maintained by means of techniques described in detail previously (Augustine and Eckert, 1984; Augustine *et al.*, 1985a). Conventional electrophysiological methods were used to stimulate the most distal "giant" presynaptic axon with extracellular wire electrodes. The connective containing the presynaptic axon usually was dissected (Augustine and Eckert, 1984) to eliminate other synaptic inputs that innervate the postsynaptic axon (Martin and Miledi, 1986), and to permit examination of transmission at the giant synapse in isolation. We often recorded postsynaptic potentials (PSPs) from the most distal giant postsynaptic axon with an intracellular microelectrode, although in some experiments, postsynaptic currents (PSCs) were measured with a two-microelectrode voltage clamp. Usually these signals were digitized (12-bit resolution) and stored on a Digital LSI-11/23+ computer system and analyzed with previously published procedures (Augustine *et al.*, 1985b). In a few experiments, signals were displayed on a storage oscilloscope and analyzed manually. Unless otherwise indicated, all results reported here were obtained in a minimum of five independent experiments.

To examine the effects of various PKC activating drugs on transmission, stock solutions of these compounds in DMSO were prepared, and these were then mixed with squid saline (enclosed in a capped polyethylene test tube and vigorously agitated with a Vortex mixer) to yield a final DMSO concentration of 0.1% or less. An identical concentration of DMSO routinely added to control salines had no obvious effect upon synaptic transmission. In some cases, stock solutions of 12-deoxyphorbol, 13-butyrate 20-acetate were prepared in 20 m*M* HEPES (pH 7.2) instead of in DMSO. Such solutions produced physiological responses indistinguishable from those obtained with solutions prepared in DMSO.

Giant synapses bathed in normal squid saline (454 m*M* NaCl, 54 m*M* $MgCl_2$, 11 m*M* $CaCl_2$, 10 m*M* KCl, 3 m*M* $NaHCO_3$, 10 m*M* HEPES buffer, pH 7.2) release so much transmitter that the postsynaptic response is sufficient to produce an action potential in the postsynaptic cell. We therefore lowered the extracellular Ca concentration to 2.2–2.8 m*M* (by equimolar substitution of $MgCl_2$ for $CaCl_2$) to reduce the amplitude of postsynaptic responses below the level of action potential generation, and to facilitate quantitative assessment of the effects of drugs upon transmission. Also in low Ca, the postsynaptic membrane potential was more easily controlled when the voltage clamp was used to measure PSCs. Under these conditions, transmission was often stable for many hours (*e.g.*, Fig. 2 of Augustine and Charlton, 1986).

Experimental solutions were delivered to the giant synapse by three different methods. Usually, solutions were delivered via a cannula inserted into the artery that irrigates the giant synapse (Augustine and Charlton, 1986). This technique permits more rapid delivery of solutions to the synapse than is possible by simply adding the solutions to the bulk medium surrounding the stellate ganglion. When using this technique, solutions often were dyed with Phenol Red to visualize the movement of solutions through the circulatory system and into the ganglion. Control experiments indicated that the effects reported in this paper were not caused by the presence of Phenol Red. In other experiments, solutions were delivered by a focal pipette delivery method (Augustine *et al.*, 1985a), or by simple addition to the bulk medium. Very similar results were obtained when kinase C activators were delivered by any of these three methods.

Results

To assess the role of PKC in transmission at the squid synapse, we tested known kinase activating agents for their ability to alter synaptic transmission. The selected agents all mimic diacylglycerol, a product of membrane phospholipid breakdown that is thought to be an intracellular messenger responsible for PKC activation *in vivo* (Kishimoto *et al.*, 1980; Nishizuka, 1984). Two classes of agents were examined. First, membrane-permeant phorbol esters, which apparently act at the diacylglycerol binding site of PKC to activate the enzyme (Bell, 1986; Kikkawa *et al.*, 1983; Nishizuka, 1984), were applied to the synapse. Second, we examined the action of a synthetic diacylglycerol, 1,2-oleoylacetylglycerol (OAG), which has a limited ability to permeate membranes and activate PKC when applied extracellularly (Kaibuchi *et al.*, 1982, 1983; Nishizuka, 1984). The structures of the compounds tested are shown in Figure 1.

DPBA enhances transmission

The actions of the phorbol ester, 12-deoxyphorbol, 13-butyrate 20-acetate (DPBA) were studied in particular detail. When DPBA was applied to the squid synapse, the amplitude and rate of rise of the postsynaptic potential (PSP) increased dramatically (Fig. 2A). The increase often was so large that the membrane potential of the postsynaptic axon reached threshold, causing an action potential to be elicited even though the synapse was bathed in low Ca saline to attenuate transmission. Measuring PSPs under such conditions proved difficult, because their peak and decay were obscured by the action potential. In such cases, we measured the initial rate of rise of the PSPs as an indicator of changes in transmission (Miledi and Slater, 1966; Llinas *et al.*, 1981; Augustine and Charlton, 1986). To reduce complications asso-

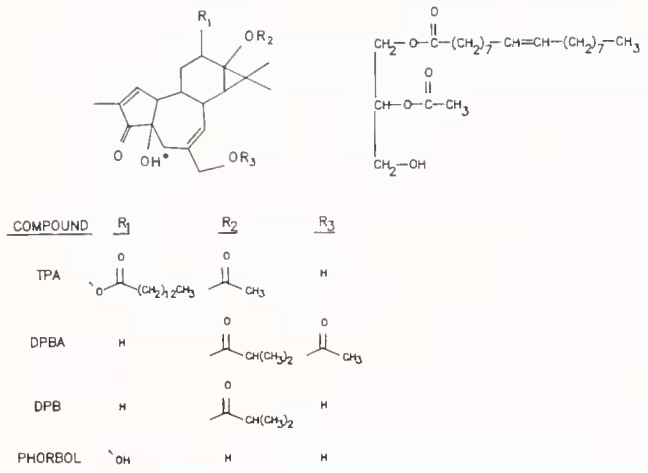

Figure 1. Structures of the compounds whose effects on synaptic transmission were examined in this study. These compounds include a variety of phorbol esters and related compounds (left) and the diacylglycerol, 1-oleoyl-2-acetylglycerol (right). The phorbol ester derivatives are tetradecanoyl phorbol-acetate (TPA), 12-deoxyphorbol, 13-butyrate 20-acetate (DPBA), 12-deoxyphorbol, 13-isobutyrate (DPB), and the phorbols, 4-α phorbol and 4-β phorbol. The structures of 4-α and 4-β phorbol are similar, except that the hydroxyl group indicated by the asterisk points down, out of the plane of the page, for 4-α phorbol but points up for 4-β phorbol. All other phorbol esters indicated have the hydroxyl group in a configuration identical to that of 4-β phorbol.

ciated with the production of postsynaptic action potentials, we often voltage clamped the postsynaptic terminal and directly measured the postsynaptic currents (PSCs) underlying the PSPs. Under such conditions, DPBA was still capable of increasing transmission (Fig. 2B), with changes in PSC amplitude being roughly equivalent to the increases in PSP amplitude produced by comparable concentrations of DPBA. Because DPBA increases PSCs as well as PSPs, DPBA does not enhance transmission solely by changing the input resistance of the postsynaptic cell.

Changes in transmission produced by DPBA usually were gradual in onset. An example of the time course of the changes in PSCs produced by DPBA, from the same experiment shown in Figure 2B, is illustrated in Figure 3. In this example, PSCs were maximally increased within 25 min of initiating DPBA perfusion. In other experiments, maximal responses were obtained at times ranging from 5 to 43 min. Part of the variability in the time course of the synaptic response to DPBA is related to the speed with which the DPBA-containing saline was delivered to the synapse via the circulatory system; *i.e.*, the speed of response could be correlated with the time required for the arrival of the tracer dye, phenol red, or high-Ca saline at the synapse. However, even after the DPBA solution had arrived at the preparation, there were delays until the maximum change in transmission occurred. Thus, the time course of the response of the synapse to DPBA treatment seems to be determined partially by the time required for DPBA to reach the giant synapse and partially by a subsequent step. Exposure of the synapse to DPBA by focal pipette delivery (Augustine *et al.*, 1985a), or addition of DPBA to the bulk solution in the recording chamber, produced similar increases in transmission, although changes in transmission were very slow when DPBA was simply added to the bath. In all subsequent experiments, drugs were delivered by arterial perfusion, unless otherwise indicated.

The reversibility of DPBA at the squid synapse was variable. In the experiment shown in Figure 3, PSC amplitude gradually recovered from DPBA treatment during a 1-h long rinse with drug-free saline. The decrease in PSC amplitude was due to a reversal of the effect of DPBA, rather than a progressive deterioration of transmission, because PSC magnitude roughly returned to its pre-treatment level and appeared to be stable for another 60 min of recording not shown on the graph. Such results were found in 7 out of 11 experiments in which stable recording conditions were maintained for more than 60 min following DPBA exposure. In the remaining experiments, the effects of DPBA did not reverse (*i.e.*, the incre-

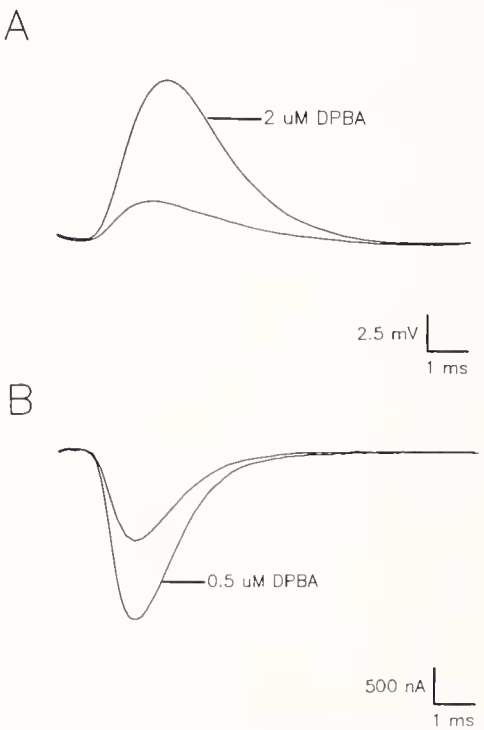

Figure 2. DPBA enhances transmission at the squid giant synapse. (A) Superimposed single traces of PSPs recorded in the absence of DPBA and after a 15-min exposure to 2 μM DPBA reveal that DPBA treatment produced a large increase in PSP magnitude. (B) Superimposed single traces of PSCs elicited in the absence of DPBA and after a 30-min exposure to 0.5 μM DPBA indicate that PSCs were increased to roughly the same extent as PSPs recorded from preparations exposed to the same concentration of DPBA.

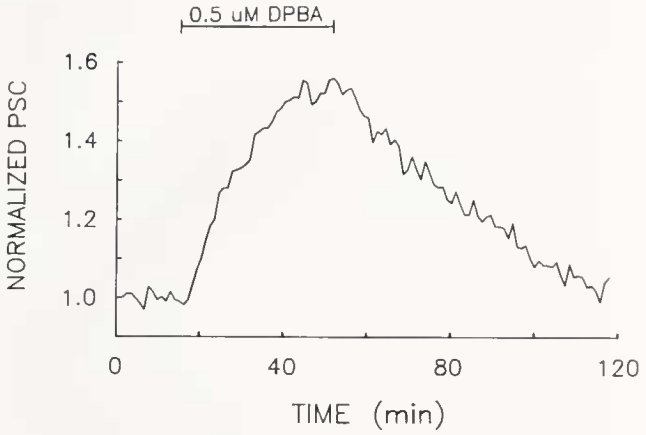

Figure 3. Time course and reversibility of DPBA effects on transmission. PSCs were elicited by stimulating the presynaptic axon every 60 s, and 0.5 μM DPBA was applied by arterial perfusion during the period indicated by the bar. DPBA produced an increase in PSC magnitude (measured by integrating each PSC record) that reached a maximum and then slowly recovered during prolonged exposure to DPBA-free saline. PSC integrals have been normalized by dividing by the mean value of the PSC integrals measured before DPBA treatment.

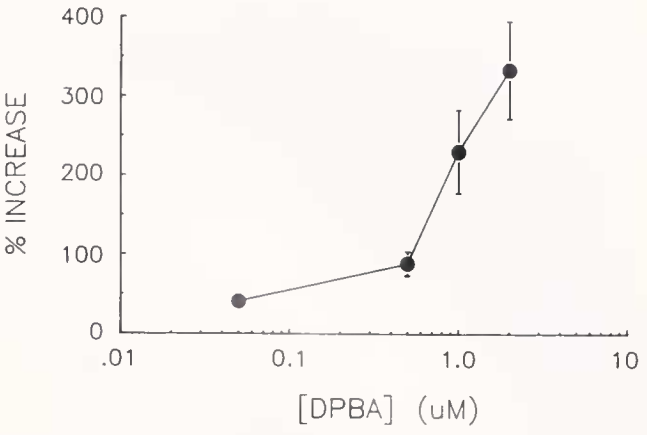

Figure 4. Concentration-dependence of DPBA effects on transmission at the squid giant synapse. Combined results of experiments examining both PSPs and PSCs reveal that DPBA concentrations as low as 50 nM increase transmission, with larger effects produced by higher concentrations of DPBA. Points are means of 4 to 12 replicates, combining measurements on both PSPs and PSCs, and error bars indicate ±S.E.M., when this value is larger than the symbol.

ment in postsynaptic response declined less than 30% after 60 min in DPBA-free saline). In these cases synaptic transmission was enhanced for hours after removal of DPBA from the saline in which the giant synapse was bathed. Such irreversibility is commonly observed with phorbol esters (*e.g.*, Publicover, 1985; Eusebi *et al.*, 1986; Shapira *et al.*, 1987; Storm, 1987) and is not unexpected, given that these molecules are hydrophobic and are also not readily metabolized within cells (Bell, 1986).

The experiments described above were performed with rather high concentrations of DPBA to optimize our ability to detect its actions on transmission. However, DPBA could enhance transmission at substantially lower concentrations. Concentrations of DPBA as low as 50 nM, the lowest concentration that we tested, increased PSP and PSC amplitude. Results of experiments with DPBA concentrations ranging from 50 nM to 2 μM are summarized in the concentration-response curve shown in Figure 4. This curve shows little sign of saturation at 2 μM, the highest DPBA concentration that we studied. Assuming that this curve can be described by a saturable function, the apparent K_D for such a function would be 1 μM or higher. Because of the hydrophobic nature of DPBA and the complex morphology of the squid stellate ganglion, we suspect that this value greatly over-estimates the actual affinity of the synapse for DPBA (see Discussion).

In summary, we find that DPBA produces concentration-dependent increases in transmission at the squid giant synapse. These observations are consistent with a role for PKC in transmission at this synapse.

Other PKC activators enhance transmission

If kinase C is involved in transmission at the squid synapse, then other PKC activators should produce changes in transmission similar to those produced by DPBA. The phorbol ester, tetradecanoyl phorbol-acetate (TPA), a compound used to activate PKC in a variety of experimental systems (Castagna *et al.*, 1982; Publicover, 1985; Caratsch *et al.*, 1986; Shapira *et al.*, 1987), was also capable of enhancing synaptic transmission (Fig. 5A). The effects of TPA upon transmission appeared similar in magnitude (Table I) and time course to those of DPBA, although the example shown in Figure 5A illustrates an

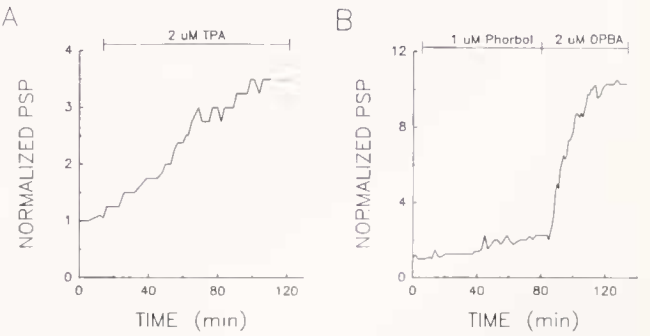

Figure 5. Other kinase C activators, such as TPA, enhance PSPs (A), while compounds that do not activate the kinase, such as 4-alpha phorbol, have little effect (B). The relatively small effect of 4-alpha phorbol is not due to an inability of the preparation to respond to phorbol esters, because subsequent treatment with 2 μM DPBA produced a robust increase in transmission. Peak amplitude of PSPs were measured in both experiments and were normalized by dividing by the mean amplitude of PSPs recorded prior to drug treatment.

Table I

Effects of various compounds on transmission at the squid giant synapse

Compound	Concentration	% Increase in synaptic response
DPBA	1 μM	229 ± 58 (n = 8)
	2 μM	321 ± 53 (n = 12)
DPB	1 μM	86 ± 9 (n = 5)
TPA	2 μM	298 ± 84 (n = 4)
4-β phorbol	1 μM	6 ± 12 (n = 10)
4-α phorbol	1 μM	1 ± 23 (n = 6)
OAG	50 μM	20 (n = 1)
	100 μM	45 ± 18 (n = 8)

Increases are expressed as mean change in PSPs or PSCs ± S.E.M.

unusually slow response to TPA. Still another phorbol ester known to activate PKC, 12-deoxyphorbol, 13-isobutyrate (Dunn and Blumberg, 1983), also increased PSP amplitude (Table I), but was less effective than DPBA. Another difference between the effect of DPB and the other kinase C activators was that, in three out of five experiments, the increase in transmission that it produced was transient. However, all three phorbol esters known to activate PKC enhanced transmission at the squid synapse.

Conversely, structurally related compounds that do not activate PKC were not able to enhance transmission. We examined two stereoisomers of the parent compound, phorbol, both of which have very weak abilities to activate PKC (Castagna *et al.*, 1982). Treatment of synapses with 1 μM concentrations of 4-α phorbol sometimes produced modest increases in transmission (Fig. 5B), but small decreases were also seen. On average, neither 4-α phorbol nor 4-β phorbol produced consistent changes in the postsynaptic response (Table I). Thus, only compounds known to activate PKC enhance transmission at the squid synapse.

To reinforce this conclusion, we tested the action of OAG, the synthetic diacylglycerol analog. OAG seemed to increase the size of evoked PSPs when applied to the synapse at concentrations of 50 μM or greater (Table I). The relatively poor efficacy of OAG, compared to the active phorbol esters, could reflect its relatively weak ability to approach and permeate synaptic membranes or the fact that phorbol esters are more potent than diacylglycerol in activating PKC (Bell, 1986). Nevertheless, the observation that OAG, too, enhances transmission strengthens the argument that activation of PKC increases transmission at the squid giant synapse. Taken together, our results obtained with six compounds suggest that activation of PKC underlies the potentiating effect of DPBA and other phorbol esters on transmission at this synapse.

DPBA enhances transmission at non-giant synapses

The postsynaptic axon of the squid synapse is innervated by at least three other presynaptic terminals, in addition to the so-called giant terminal that has been the subject of this and many other physiological studies (Young, 1939; Martin and Miledi, 1986). In some experiments, these other presynaptic inputs could be unambiguously identified and selectively stimulated. We could then ask whether the effects of PKC activators described here are restricted to the giant synapse, or are a more general feature of synapses in the squid nervous system.

An example of an experiment in which the activity of both the giant and another synapse were examined is shown in Figure 6. In this experiment, an extracellular stimulus applied to the connective innervating the stellate ganglion evoked two temporally dispersed PSCs. Examination of the electrical activity of the "giant" presynaptic terminal with an intracellular microelectrode (lower trace in Fig. 6) showed that the earlier of the two PSCs had the appropriate synaptic delay and other features characteristic of the PSC produced by the giant synapse. Addition of 0.5 μM DPBA caused this PSC (single arrow) to approximately double in amplitude, as expected (*e.g.*, Fig. 2B). In addition, DPBA caused an even more substantial increase in the amplitude of the later, non-giant PSC (double arrows). In four other experiments, we were able to evaluate the effects of various concentrations of DPBA upon transmission at these other synapses, usually in preparations in which the giant

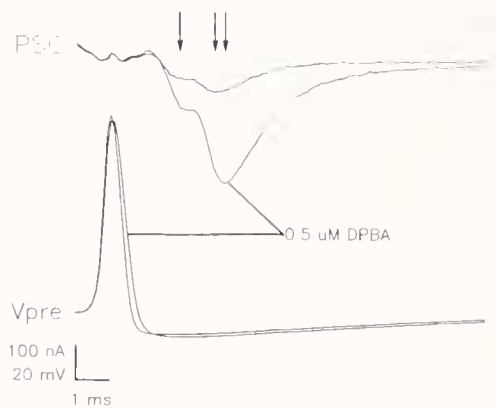

Figure 6. DPBA also enhances transmission at other squid synapses. In this experiment, extracellular stimulation of the viscero-stellate connective activated both the giant presynaptic cell (V_{pre}) and another synaptic input on to the postsynaptic axon. Both the PSC produced by the giant input (single arrow) and the non-giant PSC (double arrows) were enhanced by 0.5 μM DPBA. The action potential of the giant presynaptic terminal also was broadened by DPBA treatment.

presynaptic input had been damaged by microelectrode impalement or dissection trauma. In every case, the postsynaptic response produced by these other inputs was enhanced by DPBA. Therefore, we conclude that PKC activation regulates transmission at both the giant synapse and other synapses on the postsynaptic axon.

Discussion

Our results demonstrate that a number of activators of PKC, including three phorbol esters and a diacylglycerol compound, enhance transmission at the squid giant synapse. The effects of these compounds were concentration-dependent and long-lasting. Because structurally related compounds incapable of activating PKC had little or no physiological effect, we propose that activation of PKC is responsible for the ability of the active compounds to increase transmission. Kinase C activators enhance transmission, not only at the squid giant synapse, but also at the frog neuromuscular junction (Publicover, 1985; Eusebi *et al.*, 1986; Haimann *et al.*, 1987; Shapira *et al.*, 1987), guinea pig ileum (Tanaka *et al.*, 1984), hippocampal slices (Malenka *et al.*, 1986, 1987) and cultured hippocampal neurons (Finch and Jackson, 1987), synaptosomes from mammalian brain (Nichols *et al.*, 1987), and other neurotransmitter-secreting systems (Wakade *et al.*, 1985; Zurgil and Zisapel, 1985). These physiological consequences of PKC activation, when combined with the presence of PKC in presynaptic terminals, point to a rather general role for PKC in transmission at chemical synapses.

The concentrations of PKC activators required to alter transmission at the squid synapse are substantially higher than the concentrations used to activate purified kinase (Nishizuka, 1984) and are somewhat higher than the concentrations ordinarily applied to isolated cells (Pozzan *et al.*, 1984; Pocotte *et al.*, 1985; Rane and Dunlap, 1986). This might cast some doubt upon our conclusion that the actions of these compounds are due to PKC activation. However, the concentrations effective at the squid synapse are comparable to those that potentiate synaptic transmission in other multicellular preparations, including hippocampal slices (Malenka *et al.*, 1986, 1987; Storm, 1987). Higher concentrations are probably required in more structurally complex tissues because these compounds non-specifically partition into hydrophobic domains (*e.g.*, connective tissue) and are partially unavailable for action upon the cell under investigation. Direct microinjection of PKC into the giant pre- and postsynaptic terminals, as done with a Ca/calmodulin-dependent protein kinase (Llinas *et al.*, 1985), would provide the most definite test of the assertion that PKC activation enhances synaptic transmission.

Site and mechanism of action of kinase C activators

Our results do not allow us to state whether the action of PKC is pre- or postsynaptic. We have eliminated one possible source, namely an increase in postsynaptic input resistance, as the sole cause of the ability of DPBA to increase transmission. However, other postsynaptic actions, such as an alteration in postsynaptic sensitivity to the transmitter (Eusebi *et al.*, 1985; Caratsch *et al.*, 1986), could contribute to the effects reported here. The unknown identity of the transmitter at this synapse, combined with the small amplitude of its single-quantal events (Miledi, 1967; Mann and Joyner, 1978; Augustine and Eckert, 1984), makes it difficult to assess postsynaptic contributions to the response. In other systems, PKC activators have been shown to increase the amount of neurotransmitter released by presynaptic action potentials (Tanaka *et al.*, 1984; Wakade *et al.*, 1985; Zurgil and Zisapel, 1985). Recent experiments suggest that DPBA also acts presynaptically at the squid giant synapse (Augustine *et al.*, 1986).

PKC activation may affect transmitter release by a variety of mechanisms. PKC could increase the calcium-sensitivity of secretion, lowering the concentration of calcium necessary to stimulate transmitter release (Knight and Baker, 1983; Knight and Scrutton, 1984; Pozzan *et al.*, 1984; Pocotte *et al.*, 1985). Because transmitter release appears very sensitive to intracellular pH (Drapeau and Nachshen, 1988), PKC activation might be altering presynaptic H^+ regulation (Moolenar *et al.*, 1984; Swann and Whitaker, 1985). PKC also may enhance mobilization of transmitter (Hochner *et al.*, 1986). Finally, because PKC activators alter transmembrane ion currents (reviewed in Kaczmarek, 1986; Miller, 1986), a change in presynaptic ion currents could underlie the effect of these agents upon release. Consistent with this possibility, preliminary results suggest that PKC activation augments transmission at the squid synapse by decreasing presynaptic potassium current and consequently broadening the presynaptic action potential (Augustine *et al.*, 1986). Such an increase in presynaptic action potential duration is evident in Figure 6. These and other possible mechanisms of PKC action at synapses merit further attention.

A potpourri of protein kinase actions

Although the squid giant synapse has long been regarded as the preparation of choice for biophysical analysis of chemical synaptic transmission, it is sometimes neglected by those interested in molecular aspects of synaptic function. Our observation that PKC activators enhance transmission at this synapse, when combined with the pronounced effects observed when a Ca/calmodulin-dependent protein kinase is injected into the pre-

synaptic terminal of the squid synapse (Llinas *et al.*, 1985), makes it clear that transmission at this synapse is regulated by several of the molecular mechanisms thought to be important in determining the efficacy of other synapses. One interesting exception is the cyclic AMP-dependent protein kinase: while agents that activate this kinase alter the function of many synapses (Standaert and Dretchen, 1979; Kandel and Schwartz, 1982), they appear to have little effect on transmission at the squid synapse (G. Augustine, M. Charlton, A. Gurney, and S. Smith, unpub.). Thus, the squid synapse appears to be an appropriate model system for further explorations of the functional role of kinase C and at least some other types of regulatory molecules.

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